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CONTACT TRANSFER OF VX FROM CONTAMINATED GRASS ONTO ARMY COMBAT UNIFORM

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14. ABSTRACT: Toxicological investigations have shown that exposure to surfaces contaminated with chemical warfare agents (CWAs) can present a contact hazard. Previously, we developed standardized protocols for determining contact transfer (exposure) of agent from contaminated soils onto Army Combat Boot soles and Army Combat Uniforms (ACUs). We adapted those protocols to determine the direct contact transfer of CWA from contaminated leaf surfaces onto ACU swatches. Grass leaves (<i>Echinochloa crus-galli</i>) from intact, living plants were individually contaminated with 1 µL of <i>O</i> -ethyl- <i>S</i> -(2-diisopropylaminoethyl) methyl phosphonothioate (VX). Post-dissemination, leaves were removed, and three layers of ACU were placed atop each contaminated leaf, so that the bottom ACU layer was in direct contact with the VX-contaminated leaf surface. The ACU layers were covered with a Plexiglas disk (0.6 cm thick × 9.8 cm diameter) to equally distribute the force resulting from central placement of a standard mass atop the disk. Total proportions of VX transferred from contaminated leaves to ACU at 0.017 (1 min), 0.25, 0.5, 1, and 4 h post-dissemination were approximately 71, 5, 0.8, 0.3, and 0.1%, respectively, of the VX disseminated per leaf. Trace amounts of VX were detected in the third layers of ACU at times 0.017 and 0.25 h post-dissemination.					
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PREFACE

The work described in this report was authorized under WBS R.0013813.81.4. The work was started in May 2014 and completed in September 2015.

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CONTACT TRANSFER OF VX FROM CONTAMINATED GRASS ONTO ARMY COMBAT UNIFORM

1. INTRODUCTION

Little scientific information exists to describe the hazard to Soldiers that is associated with chemical agent–plant interactions. Without a more complete understanding of these interactions, it is difficult to predict the persistence of the potential hazard posed by agents in the environment, specifically, that arising from the contact transfer of chemical warfare agent (CWA) from contaminated plant foliage. Toxicological investigations have shown that exposure to surfaces contaminated with chemical agent presents a contact hazard (Manthei et al., 1986, 1988). Because the chemical agent *O*-ethyl-*S*-(2-diisopropylaminoethyl) methyl phosphonothiolate (VX) has a low vapor pressure, contact with contaminated surfaces is considered to be a primary route of exposure for Soldiers.

Contact transfer (exposure) testing measures the amount of contamination on a surface that transfers, under standardized, representative conditions, from a contaminated surface onto a transfer material of interest. In conjunction with toxicity data, contact transfer (exposure) results can predict contact hazards from the transmission or migration of toxic materials to skin. Various materials, such as acrylic, glass, stainless steel, painted steel, concrete, cloth, and dental dam, have been used by researchers as transfer materials to determine residual toxicity (Manthei et al., 1986, 1988). In those studies, transfer materials were contaminated with agent and then decontaminated by rinsing. The resulting transfer materials were placed directly onto rabbit skin. Studies have also been performed whereby transfer materials were placed on rabbits before the outer surface of the transfer material was contaminated (D’Onofrio, 2013). For all of these studies, the objective was to use the rabbits’ toxic responses to determine the amount of chemical agent and time (post-dissemination) that are required for the agent to penetrate the material.

In research conducted in the late 1950s (Reich, 1959a, 1959b), field plots composed of mostly grasses were contaminated with VX. A roller covered with material was then traversed through the field (simulating a crawling Soldier) to estimate the contact transfer from a VX-contaminated field to a Soldier. Blotting samplers were also used to estimate the amount of VX transferred through contact. However, in these studies, variables that are inherently unstable in ambient field conditions were not adequately controlled. Ground temperatures ranged from 12 to 28 °C and relative humidity ranged from 38 to 81%, thereby resulting in highly variable data. Other studies have shown that weather conditions can affect the persistence of agent in the environment (Reich, 1960).

In previous studies, we developed procedures to reliably determine contact transfer of chemical agents from soil directly onto Army Combat Uniform (ACU). Standard mass (\times gravity) was used as the force to produce a standard measure of exposure potential. For the work reported here, we adapted these methods to study the contact transfer of chemical agents that were disseminated onto living, intact plants within chemical surety hoods under controlled conditions, from grass foliage to ACU.

We investigated the amount of VX transferred from contaminated *Echinochloa crus-galli* grass (commonly referred to as barnyard grass) directly onto ACU under standardized, controlled conditions over a range of representative times post-dissemination. The information gained from this study, in conjunction with measurements from mammalian toxicity testing, will be useful in understanding and modeling the hazards associated with contacting VX-contaminated foliage.

2. METHODS

2.1 Chemical

The CWA used in this study was VX (93% purity; Chemical Agent Standard Analytical Reference Material [CASARM] grade; Chemical Abstracts Service [CAS] no. 50782-69-9). VX was stabilized with diisopropylcarbodiimide (5% by weight; CAS no. 693-13-0; Sigma-Aldrich; St. Louis, MO). Reagent-grade isopropyl alcohol (IPA; CAS no. 67-63-0; Sigma-Aldrich) was used in solvent extraction.

2.2 Plant Selection and Culture

We selected the grass species *E. crus-galli* for this investigation. *E. crus-galli* is one of the most prevalent natural grass species worldwide. It is tolerant of dry and wet natural habitats, and it provides forage for grazing animals as well as food and habitat for wildlife (USDA NRCS 2016). Methods were developed to enable *E. crus-galli* plant growth within a chemical agent surety hood, as described in detail by Simini et al. (2016).

2.3 Transfer Material

We used ACU as our transfer material (Table 1), which was obtained from the U.S. Army Natick Soldier Research, Development and Engineering Center (Natick, MA). To replicate battlefield condition, the ACU material was laundered four times in accordance with American Association of Textile Chemists and Colorists (AATCC; Research Triangle Park, NC) Test Method 135. Laundering included the use of AATCC Standard Reference Detergent 124, a 140 °F wash temperature, an 80 °F rinse temperature, and automatic drying on the permanent press setting. Swatches were cut from the laundered ACU to use as transfer material. ACU swatches (2.5 × 3.2 cm) were cut using an Olfa 60 mm Quick Change rotary cutter (model RTY3/NS; Olfa Corporation; Osaka, Japan).

Table 1. ACU Material Specifications*

In accordance with MIL-DTL-44436A, Class 8
Wind-resistant poplin
50%–50% nylon–cotton ripstop blend
Wrinkle-resistant finish
Desert camouflage pattern

* From MIL-DTL-44436A, *Detail Specification: Cloth, Camouflage Pattern, Wind Resistant Poplin, Nylon/Cotton Blend.*

2.4 Dissemination of Agent Droplets onto Leaves

Plant stands were constructed to hold the pots in fixed positions, and a Petri dish was placed under each pot (Figure 1). Each pot was secured to a ring stand with an adjustable ring clamp. Individual *E. crus-galli* leaves were secured in a horizontal position before VX dissemination to prevent uncontrolled deposition of agent during and after dissemination. Plant leaves were laid horizontally across a ring near the top of the plant canopy and secured to the ring by lengths of clear plastic (cellulose acetate) tape folded onto itself and placed across the leaf surface, thus preventing the sticky adhesive from coming into contact with the leaf surface. The ends of the folded tape were secured to the ring with additional tape while maintaining slight pressure on the leaf surface (Figure 2). This method of securing individual leaves in a horizontal position prevented any possible leaf surface damage caused by tape removal. It also ensured that the disseminated agent droplets contacted the leaf surface at the intended point, and that those locations could be easily identified for further investigation.

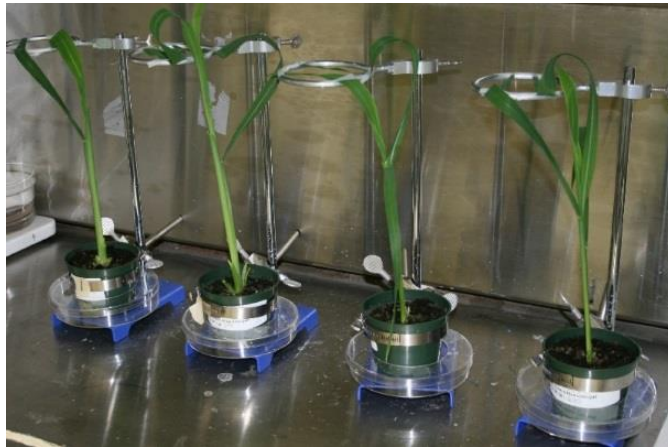


Figure 1. *E. crus-galli* plants with plant stands in the surety hood.



Figure 2. Clear plastic tape was used to secure *E. crus-galli* leaves in a horizontal position on the plant stands.

A Hamilton Gastight syringe (Hamilton Company; Reno, NV) was used to apply a single 1 μL VX droplet (for all experiments, average droplet size was 0.9059 ± 0.0335 mg) onto each secured plant leaf. One droplet was applied to each single leaf to prevent the merging of droplets (which might confound subsequent measurements). The leaves remained attached to the plants until predetermined times after agent dissemination, when each leaf was removed and used for the contact transfer procedures. During each experiment, at intervals during the dissemination of agent onto foliage, individual replicate reference droplets of VX were placed in sample vials. The amount of VX in each replicate droplet was analytically determined for quality assurance and control purposes. The mean VX values for the respective reference droplets were used in calculations to determine the percentages of VX that transferred from the foliage onto the ACU. The surety hood was equipped with two LumiBar LED light strips (LumiGrow, Inc.; Emeryville, CA). The canopy light intensity was 300 to 350 $\mu\text{mol s}^{-1} \text{m}^{-2}$, as measured with an MQ-200 Quantum sensor equipped with an AM-310 sensor wand (Apogee Instruments; Logan, UT). The temperature within the surety hood was maintained at 22 ± 2 °C, and the relative humidity was maintained at $50 \pm 10\%$. The average airflow through the hood was 1.50 ± 0.09 mph, as measured at the face of the hood using an AirData multimeter (ADM-870C; Shortridge Instruments, Inc.; Scottsdale, AZ).

2.5 Contact Transfer Procedure

In separate experiments, single 1 μL VX droplets were disseminated onto individual plant leaves. The VX droplets were allowed to equilibrate on the leaves for 0.017, 0.25, 0.5, 1, and 4 h before individual leaf replicates were removed from the grass plants and immediately subjected to contact transfer experiments.

When the leaf replicates were placed on a solid surface, the midrib veins on the ventral sides of the leaves were large enough to prevent the even distribution of applied force during the contact transfer experiments. Therefore, instead of a solid surface, we used a sand media bed to conduct the experiments on. When the ventral sides of the leaves were pressed into

the sand, the applied force on the adaxial (upper) side could be uniformly distributed across the leaf. Air-dried medium sand (as described in the appendix) was placed in glass Petri dishes (10.0×2.0 cm), and the sand was settled by tapping the Petri dishes on the benchtop. A stainless steel screed was applied to remove excess sand and level the sand surface with the top edge of the dish (a 2.0 cm depth).

To determine whether the disseminated VX droplet was soaking through the leaf, two pieces of M8 indicator paper were placed on the sand bed under the leaf. A space of approximately 5 mm was left between the two M8 strips, which was sufficient to allow the veins to depress into the sand. At a predetermined time post-dissemination, the VX-contaminated leaf was removed from the plant by using forceps to hold the leaf approximately 3 in. (7.5 cm) from the end closest to the stem. The leaf was then cut next to the forceps (on the side of the forceps nearer the stem). The leaf was placed onto the sand bed, and the central midrib vein was positioned in the space between the M8 indicator papers (Figure 3A). Layers of ACU (three swatches, each 2.5×3.2 cm) were placed over the contaminated area of the leaf, and a Plexiglas disk (0.6 cm thick \times 9.8 cm diameter; Laird Plastics; Baltimore, MD) was placed atop the ACU material (Figure 3B). A standard mass (1000 g) was centrally placed atop the Plexiglas disk (Figure 3C) to equally distribute the force resulting from the standard mass \times gravity. The stack of ACU swatches remained in contact with the contaminated leaf for 10 min and was then removed. Each swatch was placed in a separate sample bottle and extracted with 2 mL of IPA for a minimum of 1 h. We used three layers of ACU to determine whether VX would penetrate beyond the first ACU layer when in direct contact with a leaf contaminated with 1 μ L of VX, and also to ensure that the VX on the contaminated leaf would not transfer through multiple layers onto the Plexiglas disk, and therefore, not be included in the analytical determination of the total amount of VX transferred.

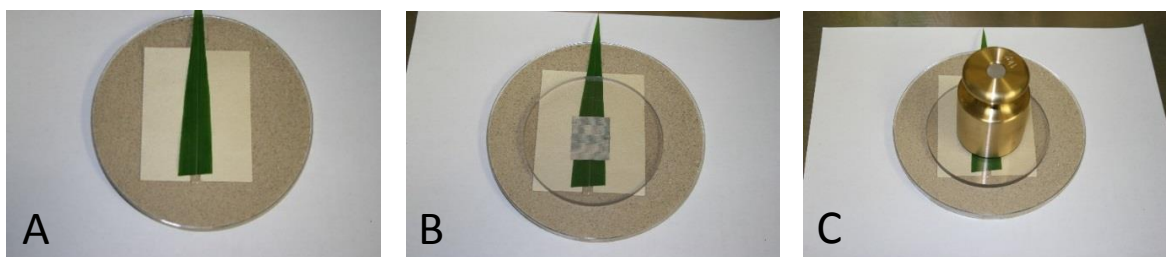


Figure 3. (A) leaf with midrib vein positioned between the M8 indicator papers; (B) leaf with applied ACU and Plexiglas disk; (C) standard mass (1000 g) applied to the Plexiglas disk, initiating the contact transfer process.

2.6 Analytical Determinations

Quantitative analytical VX determinations were conducted using an Agilent 6890 gas chromatography (GC) system equipped with a flame photometric detector (Agilent Technologies; Santa Clara, CA). Quantification was achieved using an Agilent DB-5 fused silica column (30 m \times 0.32 mm, 0.5 mm film thickness). A 1 μ L sample volume was injected into the GC system using an Agilent 7683B series autosampler. The sample inlet temperature was maintained at 225 $^{\circ}$ C in splitless mode. The initial oven temperature was 80 $^{\circ}$ C, with a

temperature ramp rate of 45 °C, to a final temperature of 300 °C. A nine-point calibration curve (0.014, 0.072, 0.14, 0.73, 1.45, 3.91, 5.81, 11.62, and 23.82 ng/μL) was used to determine VX concentrations in ACU extracts. The coefficient of determination (r^2) for linear regression of the standard curve throughout these studies was $r^2 = 0.9995$ (± 0.0003). The instrument limit of detection was 0.005 ng/μL, based on peak-to-peak background noise for this method.

Quantitative analytical determinations of VX, for low levels of VX and confirmation of GC results, were conducted using high-performance liquid chromatography linked with tandem mass spectrometry (HPLC–MS/MS) with an Agilent 1260 liquid chromatography (LC) triple-quadrupole mass spectrometer and MassHunter data acquisition and analysis software (Agilent). The HPLC system was fitted with an Agilent Eclipse XDB-C₁₈ column (5 μm, 4.6 × 150 mm). Sample injections were 1 μL. A 13 min separation method was used; the composition of mobile phase A was 0.1% formic acid (v/v) in water, and mobile phase B was 0.1% formic acid (v/v) in methanol. The gradient conditions used for LC separation are presented in Table 2.

Table 2. HPLC Gradient for VX Quantitation

Time (min)	Mobile Phase (%)	
	A	B
0	99.9	0.1
2	99.9	0.1
7	5	95
8	5	95
11	99.9	0.1
13	99.9	0.1

The HPLC column eluent was delivered to an electrospray ionization source maintained in positive ion mode. MS/MS discrimination was performed via the multiple reaction monitoring (MRM) technique, incorporating isotope dilution (VX- d_5), using three mass transitions: VX quantitation, VX confirmation, and VX- d_5 internal standard (Table 3).

Table 3. MRM Mass Transitions

Analyte	Mass (Da)	
	Precursor	Product
VX- d_5 internal standard	273	128
VX quantitation	268	128
VX confirmation	268	86

Calibration was conducted by plotting the relative responses of VX and VX- d_5 as functions of concentration. An 11-point calibration curve (5.0–5000 pg/μL of VX, each with 50 pg/μL of VX- d_5) was used to construct a linear calibration curve (1/x weighting). All analyzed samples were prepared to contain 50 pg/μL of VX- d_5 (internal standard), and reported

VX concentrations were calculated by application of the equation of fit and dilution factors, as applicable. The instrument limit of detection was 0.5 pg/ μ L, based on the peak-to-peak background noise for this method.

2.7 Determination of Appropriate Mass to Apply

The standard flat-headed, stackable masses that were used in developing the protocols for this method were obtained from Fisher Scientific (Suwanee, GA) and Central Carolina Scale (Sanford, NC). The efficacy of a net standard mass applied to ACU was determined to produce standardized measures of exposure. As described in Section 2.5, a 1 μ L droplet of VX was disseminated onto an intact grass leaf, and at 0.017 h (1 min) post-dissemination, the leaf was removed from the plant and placed onto sand-bed media. ACU swatches (three layers) were placed over the contaminated site and covered with a Plexiglas disk, and a standard mass was applied. The net standard masses tested were 250, 500, 1000, 1500, and 2000 g, with four replicate samples ($n = 4$) determined per net standard mass tested. The three swatches remained in contact with the contaminated leaf for 10 min. The swatches were then removed, separated, and extracted individually for a minimum of 1 h with 2 mL of IPA, to determine the relative efficacy of each net standard mass in the contact transfer process.

2.8 Determination of Appropriate ACU Contact Time on Agent-Contaminated Leaves

We determined the appropriate amount of exposure time that the ACU swatches should remain in contact with the contaminated leaf. As described in Section 2.5, after a 1 μ L droplet was disseminated onto a leaf and allowed to equilibrate for 0.017 h (1 min), the leaf was removed from the plant and placed on a sand bed, three layers of ACU were placed over the VX droplet site, a Plexiglas disk was applied to the ACU, and a 1000 g standard mass was placed atop the Plexiglas disk. The contact times of 5, 10, and 15 min were independently tested, and each was replicated a minimum of four times ($n \geq 4$). At the conclusion of each contact time, the ACU swatches were removed, and each was extracted separately for a minimum of 1 h with 2 mL of IPA.

2.9 Statistical Analyses

The 95% confidence level was used in all calculated determinations of statistical significance. Analysis of variance (ANOVA) and Fisher's least-significant difference (LSD) means comparison test were conducted on untransformed data. *Systat*, version 11 (Systat Software, Inc.; San Jose, CA) was used to perform the statistical calculations.

3. RESULTS

3.1 Applied Mass

The amount of VX transferred from a contaminated leaf (1 μ L VX droplet) by contact resulting from a 1000 g standard mass, applied to the Plexiglas disk covering the ACU swatches, was significantly greater (probability [p] ≤ 0.05) than that from the respective forces resulting from either the 250 or 500 g standard masses \times gravity. Standard masses greater than

1000 g did not result in significantly greater ($p > 0.05$) amounts of VX transfer from the contaminated leaf to the ACU (Table 4). Therefore, the 1000 g standard mass was selected for the method protocol for definitive determinations of contact transfer (exposure) onto ACU from agent-contaminated plant foliage.

Table 4. Preliminary Testing of Standard Masses Applied to ACU in Direct Contact with VX-Contaminated Grass Foliage*

Applied Standard Mass (g)	Proportion of VX (%) Transferred to ACU [†] (\pm SD)
250	54.9 (8.2) ^a
500	59.7 (8.0) ^a
1000	70.8 (5.3) ^b
1500	73.4 (7.7) ^b
2000	75.2 (5.8) ^b

SD, standard deviation.

* Contact time, 10 min.

[†] Letters that are different indicate significant difference ($p \leq 0.05$) among the percentages of VX transferred to ACU, as determined by ANOVA and Fisher's LSD means comparison test.

3.2 ACU Contact Time

The contact time for obtaining definitive determinations of contact transfer (exposure) from agent-contaminated plant foliage onto ACU was identified by placing ACU swatches (three layers) onto individual contaminated grass leaves for 5, 10, and 15 min, respectively, in separate replicated experiments ($n = 4$), using an applied standard mass of 1000 g. There were no significant differences ($p > 0.05$) between contact times with respect to the proportionate amounts of VX transferred onto the ACU after 5, 10, and 15 min, respectively (Table 5). We selected the 10 min contact time for conducting definitive testing.

Table 5. Determination of Contact Time

Contact Time (min)	Applied Standard Mass (g)	Total VX Recovered,* % of VX Disseminated (\pm SD)
5	1000	70.4 (5.1)
10		70.8 (5.3)
15		70.7 (3.3)

SD, standard deviation.

* No significant differences ($p > 0.05$) were found between the mean percentages of VX recovered, as determined by ANOVA and Fisher's LSD means comparison test.

3.3 Agent Transferred to ACU

The average mass of VX disseminated as a single 1 μ L droplet onto individual grass leaves throughout all contact transfer testing was 0.9059 ± 0.0335 mg. Results from replicated definitive testing indicated that the proportion of VX transferred per leaf onto the ACU swatches from contaminated grass leaves at 1 min (0.017 h) post-dissemination was approximately 71% of the VX disseminated ($70.8 \pm 5.3\%$; 0.605 ± 0.045 mg) (Table 6). At 0.25 h post-dissemination, the VX transfer by direct contact was approximately 5% of the VX disseminated ($5.1 \pm 2.3\%$; 0.045 ± 0.021 mg). At 0.5 h post-dissemination, the VX transfer by direct contact was approximately 1% of the VX disseminated, and at later times post-dissemination (1 and 4 h), the VX transfer by direct contact was substantially $<1\%$ of the VX disseminated. The respective amounts of VX that were absorbed into the second layers of ACU at time points of 0.017, 0.25, 0.5, 1, and 4 h post-dissemination were $<1\%$ of the total VX disseminated. Trace amounts of VX were detected in the third layers of ACU at time points 0.017, 0.25, and 0.5 h post-dissemination (Table 6).

Under field conditions, leaf tissues can be damaged by troop movement over contaminated areas, and this could potentially cause the release of additional agent from contaminated leaves. To test this hypothesis, after completion of the direct contact transfer process, at 4 h post-dissemination, each leaf ($n = 4$) was placed into a glass Petri dish, and one swatch of ACU was placed over the droplet site. A new sample vial was then used to apply pressure to each ACU swatch, which remained in contact with the contaminated leaf, in a back-and-forth, leaf-grinding motion. The additional amount of VX transferred onto the fresh ACU swatch averaged approximately 0.015 mg, which represents an additional 1.6% of the amount of VX that was initially disseminated (Table 6).

Table 6. Contact Transfer of VX from Grass Foliage onto ACU

Time Post-Dissemination of Agent onto Leaf (h)	VX Transferred by Direct Contact with Grass (mg)			Total VX Transferred (mg) by Contact from Foliage onto ACU (\pm SD)	Total Proportion of VX Transferred (%) from Foliage by Contact* (\pm SD)
	Onto First Layer of ACU	Onto Second Layer of ACU	Onto Third Layer of ACU		
0.017	0.6005	4.6×10^{-3}	4.5×10^{-5} ⁱ	0.605 (0.045)	70.8 (5.3) ^a
0.25	0.0443	7.59×10^{-4}	4.76×10^{-5} ⁱⁱ	0.045 (0.021)	5.1 (2.3) ^b
0.5	0.0075	8.13×10^{-5}	3.2×10^{-5} ⁱⁱⁱ	0.008 (0.005)	0.8 (0.6) ^c
1	0.0025	1.14×10^{-5} ^{iv}	BDL	0.002 (0.001)	0.3 (0.1) ^c
4	0.0007	BDL	BDL	0.0007 (0.0007)	0.1 (0.1) ^c
4 ^v	0.0147	NC	NC	0.0147 (0.004)	1.6 (0.4) ^b

Notes: A single 1 μ L droplet of neat VX was disseminated onto an intact, live grass leaf and allowed to equilibrate on the leaf for a predetermined time post-dissemination. Three layers of ACU were used as the transfer material, with the first layer of ACU in direct contact with the contaminated leaf. A 1000 g standard mass was applied for 10 min exposure. Means shown for VX transferred are averages for four replicates ($n = 4$).

BDL, below detection limit.

NC, not conducted.

SD, standard deviation.

* Same letter at respective values indicates no significant difference ($p > 0.05$) among the percentages of VX transferred to ACU, as determined by ANOVA and Fisher's LSD means comparison test. Percentages were calculated using the means for VX transferred from individual contaminated leaves post-dissemination versus the means of the VX reference droplets for those individual leaves.

ⁱ VX penetrated into the third layer of ACU (two out of four replicates).

ⁱⁱ VX penetrated into the third layer of ACU (four out of four replicates).

ⁱⁱⁱ VX penetrated into the third layer of ACU (one out of four replicates).

^{iv} VX penetrated into the second layer of ACU (two out of four replicates).

^v Leaf replicates individually crushed after contact transfer was completed, 4 h post-dissemination, then contact transfer was again conducted using a fresh ACU swatch.

4. DISCUSSION

We determined the amount of agent that was transferred at various times post-dissemination onto ACU swatches in contact with an *E. crus-galli* grass leaf that had been contaminated with a 1 μ L droplet of VX (0.9059 ± 0.0335 mg). The amount of VX transferred to the ACU swatches at 1 min post-dissemination was 0.605 mg, which corresponds to a contact transfer of 71% of the VX that had been disseminated onto the grass. At 1 h post-dissemination, the amount of VX transferred to the ACU swatches was 0.002 mg, which corresponds to a contact transfer of approximately 0.3% of the VX disseminated. Exposure to VX-contaminated grass at 1 h post-dissemination, rather than 1 min post-dissemination, resulted in a 99.6% reduction in the proportion of agent that was transferred by direct contact. At 4 h post-dissemination or later, $\geq 99.8\%$ reduction occurred in the net proportion of agent transferred by contact with contaminated grass, as compared with the proportion of agent that was transferred at 1 min post-dissemination.

Similar trends were observed during open-air field studies conducted on Carroll Island, MD, in 1958 (Reich, 1959a), where VX was aerially disseminated onto sod plots (at a concentration of 2.2–13.9 g/m²) and then monitored for persistence. Persistence was examined

by blotting the contaminated areas at 1, 24, and 48 h post-dissemination. The blotting material was extracted with IPA, and the extracts were analyzed. Results showed that the proportionate amount of VX transferred to the blotter at 1 h post-dissemination was approximately 20% ($19.3 \pm 5.5\%$) of the initial VX concentration. At 24 and 48 h post-dissemination, the proportionate amounts of VX transferred had decreased to approximately 1% ($\pm 0.2\%$) and 0.07% ($\pm 0.02\%$), respectively, of the initial VX concentration. However, the authors noted that precipitation occurred post-dissemination, during both the 1–24 h and the 24–48 h intervals. Reich conducted additional studies on Carroll Island in 1959, wherein open-air grass plots were contaminated with VX and then traversed with a roller covered in cotton material (Reich, 1959b). Results of those studies showed that at 24 h post-dissemination, the amount of VX transferred to the roller was approximately 97% less than the amounts that were picked up when the grass was traversed within 1 h post-dissemination. In all of these Carroll Island studies, although variables such as temperature, relative humidity, and precipitation could not be controlled and replication was minimal, those results exhibited a trend that was similar to the results we obtained from the studies reported herein, which were conducted under controlled, well-focused laboratory conditions.

Ballard et al. (1968) immersed the stems and leaves of 10 day old oat seedlings in an aqueous solution of VX (10 mg/mL) for 10 s. They then monitored the amount of VX that was removed (as a function of time post-exposure) by rinsing the leaves with petroleum ether, which is a solvent for waxes, oils, and fats (CDC, 1977). They found that after 4 h post-exposure, the amount of VX residue removed from the plant leaves by the solvent wash was reduced by approximately 97%, as compared with the amount removed by solvent immediately after immersion.

The U.S. Environmental Protection Agency (USEPA) recommends reentry intervals for agricultural workers who use pesticides. The reentry intervals are based on the available toxicity data, concentrations of chemicals used, environmental conditions, crops being treated, and frequency of treatment (Watson et al., 1992). The USEPA recommends a minimum reentry interval of 48 h for workers using some of the more toxic organophosphate pesticides. State regulators are free to set more stringent intervals. Watson suggested using the Rapid Screening Hazard (RASH) method to determine the reentry interval for individuals working with VX (Watson et al., 1992). The RASH method is based on the determination of a relative potency factor (RPF), which is derived from the ratio of toxicity data from a reference chemical with an established reentry interval to toxicity data of a chemical with an undetermined reentry interval. The respective RPF values can then be used to establish a reentry interval. Using the RASH method, the reentry interval for VX would be 60 to 90 days.

Based on the results presented in this report, the RASH method that uses RPF values for pesticide exposure of agricultural workers appears to be unrealistic for extrapolating to the exposure of Warfighters under battlefield conditions. In our studies, multiple layers of ACU were used in definitive, direct, standardized contact transfer testing to determine whether VX would penetrate beyond the layer of ACU that was in direct contact with a grass leaf contaminated with a 1 μ L VX droplet. At 0.017 and 0.5 h post-dissemination, contacting the contaminated leaf for 10 min produced VX penetration into all three ACU layers; however, at 4 h post-dissemination, VX was only detected in the ACU layer that was in direct contact with

the contaminated leaf (approximately 0.1% of the VX initially applied). In a study by Simini et al. (2016), a 1 μ L droplet of VX visually appeared to be fully absorbed into a grass leaf by 4 h post-dissemination. On the basis of these two results for VX, we anticipated that full absorption into leaves should reduce the proportion of contaminating agent that is immediately available for direct contact transfer. Results from the present study confirmed that when VX droplets (1 μ L) equilibrate on grass leaves for ≥ 4 h, compared with equilibrating for 1 min, direct contact transfer of VX onto ACU is reduced by 99.9%. However, we also found that if additional pressure is applied with a twisting force to the same contaminated leaf, emulating direct contact in the field by a Soldier's knee or palm of hand, additional VX is released from plant leaf tissues even 4 h post-dissemination, thereby increasing the potential hazard. This type of rugged contact with VX-contaminated foliage at 4 h post-dissemination increased the average proportion of VX that was transferred onto the ACU by more than an order of magnitude. The effect of rugged contact or contact with damaged VX-contaminated foliage yielded exposure that approximately corresponded with that expected from direct, standardized contact transfer as early as 0.25 h (15 min) post-dissemination (95% statistical confidence; $p > 0.05$).

Based on a 70 kg soldier, the percutaneous dose that is lethal to 50% of test subjects (the LD₅₀ value) for VX is 3 mg (*Safety Data Sheet*, 2015). Potential exposure by standardized, direct contact transfer to a Soldier's ACU at 1 min post-dissemination from one grass leaf contaminated with one 1 μ L droplet of VX was approximately fivefold less than the estimated human percutaneous LD₅₀ value. However, when an otherwise unprotected ACU comes into direct contact with contaminated foliage that bears the equivalent of five or more 1 μ L droplets of VX at 1 min post-dissemination, the resulting exposure is expected to be greater than the estimated human percutaneous LD₅₀ value, based on the results of the standardized testing reported herein. Such equivalent exposure may occur as a result of contact with a greater number of grass leaves contaminated at this level (1 μ L droplet of VX per grass leaf) or simply by contact with grass contaminated at greater levels of VX (e.g., more than one 1 μ L droplet per grass leaf, or droplets that are >1 μ L in size on foliage). At 0.25 or 0.5 h post-dissemination, direct exposure that is equivalent to approximately 67 or 375 droplets (1 μ L), respectively, of VX on foliage is expected to exceed the estimated human percutaneous LD₅₀ value. If the VX has equilibrated on *E. crus-galli* grass foliage for ≥ 1 h, then direct contact via an otherwise-unprotected ACU that is equivalent to 1500 or more 1 μ L droplets would be needed to equal or exceed the estimated percutaneous LD₅₀ value. Thus, the extent of the VX-contaminated area that must be traversed should be considered by commanders making decisions regarding hazards related to Warfighter exposure to agent on the battlefield. However, the passage of time post-dissemination of VX relatively quickly reduces potential imminent hazard. Additionally, persistence of CWA in the field can be influenced by the type of foliage (Sanyal et al., 2006), and it is also a function of the CWA properties, the environmental conditions, and the epicuticular waxes and cuticles of the contaminated plants (Deseret Test Center, 1980; Gorzkowska-Sobas, 2013; Simini et al., 2016).

5. CONCLUSIONS

The standardized contact transfer method for determining agent transfer from direct contact with CWA-contaminated foliage was adapted from methods and protocols for contaminated soil. Use of this method allows for standardized comparisons of chemical agents on plant foliage and the respective corresponding hazards. The results of these focused standardized contact transfer tests, conducted under controlled environmental conditions, comport with the trends and range of results reported from open-air field studies that were conducted decades ago.

From this work, the following conclusions can be drawn:

- The proportionate amount of agent available for contact transfer from VX-contaminated *E. crus-galli* grass leaves 1 min post-dissemination was approximately 71% of the total amount of VX disseminated onto the foliage.
- When VX had equilibrated on grass foliage for 1 h or more, the proportionate amount of directly transferable VX was reduced to <1% of the initial amount of VX disseminated onto the foliage.
- At 0.017 (1 min) and 0.5 h post-dissemination, direct contact with a grass leaf contaminated by a single 1 μ L droplet of VX resulted in the penetration of VX into three layers of ACU.
- At 4 h post-dissemination, direct contact with a grass leaf contaminated with a single 1 μ L droplet of VX resulted in transfer that was detected in only the first layer of ACU.
- When VX had equilibrated on *E. crus-galli* grass foliage for 1 to 4 h, the proportionate amount of directly transferable VX was reduced to 0.4 to 0.1% of the respective initial amount of VX that was disseminated onto the foliage.
- Rugged contact with contaminated grass foliage 4 h post-dissemination increased the proportion of VX transferred onto ACU by more than an order of magnitude, compared with the proportion that was measured from direct, standardized contact transfer, thereby increasing imminent hazard.

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ACRONYMS AND ABBREVIATIONS

ACU	Army Combat Uniform
ANOVA	analysis of variance
CAS	Chemical Abstracts Service
CWA	chemical warfare agent
GC	gas chromatography
HPLC	high-performance liquid chromatography
IPA	isopropyl alcohol
LC	liquid chromatography
LD ₅₀	dose that is lethal to 50% of test subjects
LSD	least-significant difference
MRM	multiple reaction monitoring
MS/MS	tandem mass spectrometry
p	probability
r^2	coefficient of determination
RASH	Rapid Screening Hazard
RPF	relative potency factor
SD	standard deviation
USDA	U.S. Department of Agriculture
USEPA	U.S. Environmental Protection Agency
VX	<i>O</i> -ethyl- <i>S</i> -(2-diisopropylaminoethyl) methyl phosphonothiolate

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APPENDIX

PHYSICAL AND CHEMICAL PROPERTIES OF MEDIUM SAND

Table A1. Properties of AFS-50 Medium Sand*

Property		Mean	Standard Error
pH		6.05	0.15
Organic matter (%)		0.0	0.00
Sand (%)		98.9	0.20
Silt (%)		0.85	0.25
Clay (%)		0.25	0.05
Texture		Sand	n/a
Particle size range (mm)		0.25–0.50	n/a
BET surface area (m ² /g)		0.234	0.002
Cation exchange capacity (cmol/kg)		2.25	0.05
K (mg/kg)		3.5	0.5
Mg (mg/kg)		3.5	0.5
Ca (mg/kg)		42.5	0.5
% Saturation of Cation Exchange Capacity	K	0.4	0.1
	Mg	1.3	0.2
	Ca	9.4	0.1
P (mg/kg)		2.0	0.0
Cu (mg/kg)		0.3	0.0
Zn (mg/kg)		0.25	0.05
Acidity (cmol/100 g)		2.0	0.0
Conductivity (mmhos/cm)		0.065	0.005
Water-holding capacity (%)		5.3	0.3

* Obtained from Warmwell Quarry, Bardon Aggregates, Southern Warmwell, Dorchester, U.K.

Notes: All values were based on soil-test results ($n = 2$) analytically determined by Agricultural Analytical Services Laboratory, Penn State University, University Park, PA; except particle size range was supplied by the manufacturer; surface area ($n = 2$) was determined by Micromeritics, Inc., Northcross, GA; and water-holding capacity ($n = 3$) was determined by ECBC Environmental Toxicology Branch.

BET, Brunauer–Emmett–Teller.

n/a, not applicable.

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